PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61L 27/00, 29/00, 31/00, 33/00, 17/00

A1

(11) International Publication Number: **WO 99/49907**

(43) International Publication Date: 7 October 1999 (07.10.99)

(21) International Application Number: PCT/GB99/01008

(22) International Filing Date: 31 March 1999 (31.03.99)

(30) Priority Data:
9806966.9 31 March 1998 (31.03.98) GB

(71) Applicant *(for all designated States except US)*: PPL THERAPEUTICS (SCOTLAND) LTD. [GB/GB]; Roslin, Edinburgh EH25 9PP (GB).

(71) Applicant *(for US only)*: DE BONO, Anne (executrix for the deceased inventor) [GB/GB]; Leicester General Hospital, Occupational Health Dept., Leicester LE5 4PW (GB).

(72) Inventor: DE BONO, David, Paul (deceased).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: GARNER, Ian [GB/GB]; PPL Therapeutics, Roslin, Midlothian EH25 9PP (GB). GERSHLICK, Anthony, Harvey [GB/GB]; Glenfield Hospital, Clinical Sciences Building, Division of Cardiology, Groby Road, Leicester LE3 9QP (GB).

(74) Agents: CHAPMAN, Paul, William et al.; Kilburn & Strode, 20 Red Lion Street, London WC1R 4PJ (GB).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: MEDICAL DEVICES TREATED TO DISCOURAGE BLOOD COAGULATION

(57) Abstract

Medical devices are provided, at least part of the surface of which a protein having the activity of Protein C or Activated Protein C is directly or indirectly attached. The devices of the invention may be vascular prostheses such as stents. The protein may be indirectly attached to the surface of the device through an intermediate layer, such as a polymeric coating. The attachment may be mechanical. Such medical devices display reduced tendency to cause blood coagulation when in direct contact with blood. The devices of the invention may find use in various systems, such as the cardiovascular system of a mammal, where such devices, in use, come into direct contact with blood.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

MEDICAL DEVICES TREATED TO DISCOURAGE BLOOD COAGULATION

The present invention relates to medical devices, the surfaces of which are treated so as to discourage blood coagulation. Specifically, the invention relates to the use of certain proteins in the preparation of such devices.

Following contact of a foreign body with blood, complex interactions with the blood are triggered. Plasma proteins adsorb themselves on depending on plasma concentration of the protein, surface charge, wetability and geometry of the material, collision probability and, most importantly, the affinity constant of the individual proteins with the foreign body. Fibrinogen displaces other 'bland' proteins increasingly because of its higher affinity constant and plays a key role in further activation of other important plasma proteins and the coagulation cascade. Fibrinogen is also responsible for starting a cellular phase of events involving activation of platelets in conjunction with von Willebrand factor, thrombospondin and fibronectin. This dynamic interplay of plasma proteins and cells lead on to the ensuing process of thrombosis and restenosis.

The coagulation cascade described above can lead to difficulties during and after surgery if that involves devices (such as surgical instruments, prostheses, etc) coming directly in contact with blood, either temporarily or permanently. If the problem of thrombogenicity is not addressed, such devices could easily lead to formation of blood clots, especially as many such devices (eg stents) are metallic.

For example, although the two main limitations of balloon angioplasty - acute occlusion and long-term restenosis - have, to a certain extent, been addressed by the

use of stents in recent times, the problem of stent thrombosis and restenosis still remains.

The biological changes leading to restenosis after percutaneous coronary angioplasty are well-documented (*Forrester, 1991; Gibbons, 1994; Mintz, 1996*). Balloon angioplasty, by achieving plaque compression and non atheromatous stretching, produces deep injury to the vessel wall. Endothelial denudation, intimal disruption and medial damage accompany plaque splitting and plaque haemorrhage. The repair processes that result involve complex inflammatory and thrombotic pathways. Activated platelets adhere to the damaged wall within seconds and what follows is a vicious cycle of platelet deposition and thrombus formation. The coagulation pathways, chiefly extrinsic, lead to the activation of clotting factors and thrombin. The accumulation of a platelet - and fibrin - rich thrombus is incorporated into the disrupted lesion. Adherent thrombin, growth factors released from activated platelets and from the smooth muscle cells (SMC) themselves effect a phenotypic change in SMCs promoting their migration through the intimal elastic lamina and their proliferation in a newly formed intimal layer. More recently, studies have shown that an elaborate extracellular matrix produced along with augmented expression of various matrix metalloproteinases in post angioplasty is the 'third wave' in restenosis processes - explaining in part the dissociation between intimal thickening and SMC replication (*Libby, 1997*). In patients undergoing angioplasty alone, these processes are further complicated by the development of early recoil and late negative remodelling.

However, the above problem can, to a certain extent, be addressed by the use of stenting. In patients receiving a stent, recoil and negative remodelling are observed to be largely absent (*Dussaillant, 1995*). Stents are shown to produce smoothing or wound edge apposition and create a circular luminal configuration. Its dilatory

function has in cases been shown to produce a further 24h circumferential expansion. Following percutaneous coronary angioplasty, restenosis rates are 30 - 40% at follow up angiography, of which 20% have clinical recurrence. In the clinical studies "STRESS" and "BENESTENT II", stenting was shown to reduce restenosis in comparison to conventional percutaneous transluminal coronary angioplasty (*Fischman, 1994; Serruys, 1994*). Incidence of restenosis post-stenting can now be taken to be 20% (possibly 15%). Even lower thrombosis rates were achieved in a group using routine intravascular ultrasound (IVUS), achieving mean Minimal Lumen Diameter (MLD) of 3.39mm. However, only 5% of patients in this study received bailout stenting (*Colombo, 1995*). A BENESTENT-type lesion is one of native vessel, of stable angina, single lesion reference size >3mm, <15mm length, non-ostial, non-bifurcational, non-restenotic with no thrombus. Post-stent MLD for STRESS and BENESTENT II were 2.49mm and 2.48mm respectively. It is fairly certain now, given the close relation between initial post-procedural MLD (acute again) and follow up MLD (late Loss), that the value of stenting lies in the 'bigger is better' principle.

All the randomised trials however have shown a recurring feature. Improvement in technique and device may have produced a trend towards reducing emergency coronary bypass grafting and similar sequelae, but there remains little change in the incidence of myocardial infarction and mortality. This reflects the unresolved problem of acute vessel closure and subacute stent thrombosis. Optimised patient selection and procedural variables will only reduce subacute thrombosis of currently available stents to between 4 and 8% during elective use. In a group of 1500 patients studied, only 12% were BENESTENT-type lesions (*Williams, 1997*). Considering the sequelae of stent thrombosis and the substantial proposition of 'non-optimal' lesions that we deal with, this represents an unacceptable complication rate.

Acute/subacute stent occlusion is a very significant problem indeed in bailout angioplasty stenting and in small vessels. Less established risk factors include periprocedural thrombus at angiography, residual dissection and the use of multiple stents. The outcome looks very serious indeed against the background of high vascular complication rates due to deep anticoagulation treatment.

Various approaches to address the problem of blood coagulation following foreign body contact have been tried.

Systemic pharmacological interventions using heparin, aspirin, dipyridamole, ticlopidine, prostacyclincs and thromboxane-A2 receptor blockers, coumadin, cholesterol-lowering drugs and ω-3 polyunsaturated fatty acids have failed to make a critical difference. This is especially vis-a-vis the haemorrhagic side effects that so often accompany potent antiplatelet and anticoagulant agents.

Various groups have explored the modification of stent surface. Examples of use of physiochemical means include polyurethane coating (*De Scheereder, 1993*), pure polymer stents (*van der Giessen, 1992*) and chemical modification with polyethylene oxide (*Bamford, 1983*). Biological means have also been employed, such as presorbtion with proteins (eg matrix components) (*van Beusekom, 1993*) and seeding with genetically engineered endothelial cells (*Dichck, 1989*). However, these various modifications were found to have their limitations.

Immobilising drugs to the surface of stents is another possibility. There are several well-recognised methods of immobilising drugs to stents. Using heparin as an example-drug of choice, it has been shown to adsorb well onto stent surfaces from a benzalkonium alcohol-heparin solution. However the downside was the erratic rate of release of heparin (*Breckwoldt, 1991*).

Negatively charged heparin molecules have also been used to bind electrostatically to surfaces with positive charges (*Grode et al, 1969; Tanazawa et al, 1973*). Heparin has also been incorporated into a permanent component of polymer coating the stent (*Jozefowicz, 1986*). However, the results of these attempts have not been satisfactory.

The most well-recognised technique is that employed in Johnson & Johnson stents that were used in the BENESTENT II trial, involving covalent bonding of the heparin molecule aldehyde group to the stent polymer amino via end point attachment. Unfortunately the rate reduction in BENESTENT II cannot be attributed solely to heparin coating. There was no control group to this effect. Relative reduction may be the result of a variety of other possibilities including better operator experience, employing high pressure balloon, achieving larger early MLD and using IVUS.

Angiopeptin is a synthetic cyclic octapeptide analogue of somatostatin that reduces neointimal hyperplasia. De Scheerder (1995) reported that local angiopeptin delivery using drug-loaded polymer-coated metallic stents resulted in a significant reduction in luminal narrowing in porcine coronaries. Forskolin, an adenylate cyclase activating drug which causes platelet disaggregation and vasodilation, incorporated into the polyurethane coating of a heat-activated temporary stent showed delay time in thrombosis (*Lambert, 1994*). However, these methods still leave scope for improvement.

A prototype methotrexate - and heparin - coated stent also failed to show any significant effect (*Cox, 1992*).

Thus there is presently no satisfactory means for rendering foreign bodies that come into direct contact with blood non-thrombogenic. If, for example, a truly non-thrombogenic coronary stent without systemic side-effects is available for use in angioplasty, this would represent a significant advance on the present state of art. As cardiologists progress towards performing intervention in more complicated clinical situations, the challenge remains to find ways of preventing such blood coagulation occurring.

The present invention relates to the use of Protein C in addressing the problem of blood coagulation caused by contact with foreign bodies. The invention is based on the observation that Protein C can be efficiently delivered locally by attachment to medical devices that come into direct contact with blood.

Accordingly, in a first aspect of the present invention there is provided a medical device to at least part of the surface of which a protein having the activity of Protein C or Activated Protein C is directly or indirectly attached.

Protein C was first described as a vitamin K-dependant, phospholipid-binding zymogen of a serum protease anticoagulant (*Stenflo,1976; Esmon et al, 1976; Seegers et al, 1976*). It circulates in human plasma at a concentration of 2-4μg ml-1. Protein C is a glycoprotein of approximate molecular weight 62,000. Fully processed Protein C molecules contain 9-carboxyglutamic acid residues. Two molecular forms of Protein C are readily detectable in plasma, a single-chain [5-15%] and a two-chain molecule [85-95%]. The latter consists of a heavy chain [260 amino acids] and a light chain [155 amino acids] linked by a single disulphide bond.

Conversion of Protein C to the active serum protease follows the formation of a 1:1 thrombin:thrombomodulin complex on the endothelial cell surface (*Kisiel et al,*

1977; Kisiel, 1979; Owen and Esmon, 1981). This event promotes several anticoagulant properties. The conformation of thrombin alters as does its activity towards fibrinogen and factor V. Following this, the preferred substrate of the complexed thrombin becomes Protein C which it activates through specific proteolysis and liberation of a 12 amino acid activation peptide from the amino terminus of the heavy chain (*Kisiel, 1979*). The resulting Activated Protein C or APC functions as an anticoagulant by degradation of the active forms of factors V and VIII *(Comp and Esmon, 1979; Walker et al, 1979; Dahlback and Stenflo, 1980*). The anticoagulation effect of APC is substantially increased by another vitamin K-dependant protein, the co-factor Protein S. Both factors V and VIII are large, single-chain glycoproteins which are converted to active co-factors by the proteolytic action of thrombin. Following the formation of factor VII:tissue factor complex, they are involved in the activation of factor X and the subsequent prothrombin to thrombin transition during the coagulation cascade of zymogenic activation, leading to clot formation. Their inactivation by APC prevents the de novo formation of thrombin and the continued generation/expansion of clots.

A number of experimental studies have indicated that Protein C may be useful clinically in the treatment of thrombosis. The anticoagulant properties of APC have been demonstrated in primate, rabbit and dog thrombosis models (*Gruber et al, 1989; Gruber et al, 1990; Gruber et al, 1991; Arnljots et al, 1994; Sakamoto et al, 1994*). Protein C has also been proposed to possess profibrinolytic activity by indirectly promoting the activation of plasminogen to plasmin. Infusion of Activated Protein C into dogs was shown to enhance fibrinolysis (*Comp and Esmon, 1981*). Anti-cell adhesion properties of APC have also been reported (*Grinnell et al, 1994*) indicating a potential explanation for the reported anti-inflammatory properties of APC, probably arising from an inhibition of platelet and endothelial cell activation. Furthermore, Protein C is purported to be a therapy with minimal risk of inducing

bleeding complications. This was supported by the administration of Protein C and APC to normal healthy volunteers (*Okajima et al, 1990*). Indeed, elevated endogenous Activated Protein C levels in both a pig model of arterial occlusion (*Snow et al, 1991*) and in patients undergoing coronary thrombolysis with streptokinase (*Gruber et al, 1993*) indicate a central role for APC in the prevention of thrombotic complications.

However, to the applicant's knowledge, it has never been shown that attachment of Protein C to a medical device is possible, nor that Protein C would retain its natural, potent anticoagulant activity *in situ* after it is attached to such a device.

The use of Protein C or Activated Protein C in the clinic would have previously necessitated the systemic delivery of the protein by infusion, which is practically and financially disadvantageous. This is especially so in view of the fact that at present Activated Protein C can only be obtained from a limited number of sources: fractionation of human plasma, recombinant Activated Protein C from cell culture and purified from the milk of transgenic animals which express human Protein C.

The present invention is based on the finding that Protein C, Activated Protein C and other molecules having the activity of Protein C or Activated Protein C can be directly or indirectly attached on the surface of a device without undue loss of activity and can exert the biological function when the device comes into direct contact with blood. The delivery of such Protein C molecules via such devices has a number of advantages. Systemic toxic effects can be avoided. The active agent can be given in doses that will not produce side-effects but still have a high effective local concentration. Effective treatment is targeted to the site required and treatment is tailored to the pathological process more easily and precisely.

Any protein having the activity of Protein C may be used in this invention. This includes the whole, glycoprotein form of Protein C. Variants and derivatives having the activity of Protein C are also included.

Variants of Protein C or Activated Protein C may consist of the particular natural amino acid sequence of Protein C or Activated Protein C, or may have an additional N-terminal and/or an additional C-terminal amino acid sequence.

Additional N-terminal or C-terminal sequences may be provided for various reasons. Techniques for providing such additional sequences are well known in the art. These include using gene-cloning techniques to ligate together nucleic acid molecules encoding polypeptides or parts thereof, followed by expressing a polypeptide encoded by the resultant recombinant nucleic acid molecule.

Additional sequences may be provided in order to alter the characteristics of a particular polypeptide. This can be useful in improving expression or regulation of expression in particular expression systems. For example, an additional sequence may provide some protection against proteolytic cleavage. This has been done for the hormone somatostatin by fusing it at its N-terminus to part of the β-galactosidase enzyme (*Itakwa et al, 1977*).

Additional sequences can also be useful in altering the properties of a polypeptide to aid in identification or purification. For example, a signal sequence may be present to direct the transport of the polypeptide to a particular location within a cell or to export the polypeptide from the cell. Different signal sequences can be used for different expression systems. Another example of the provision of an additional sequence is where a polypeptide is linked to a moiety capable of being isolated by affinity chromatography. The moiety may be an antigen or an epitope and the affinity column

may comprise immobilised antibodies or immobilised antibody fragments that bind to said antigen or epitope (desirably with a high degree of specificity). The fusion protein can usually be eluted from the column by addition of an appropriate buffer.

Additional N-terminal or C-terminal sequences may, however, be present simply as a result of a particular technique used to obtain the protein of the present invention and need not provide any particular advantageous characteristic.

The use of proteins having one or more amino acid substitutions, deletions or insertions compared to the natural sequence of Protein C or Activated Protein C are also within the scope of the invention.

The skilled person will appreciate that various changes can sometimes be made to the amino acid sequence of a polypeptide which has a particular activity to produce variants (often known as "muteins") which still have said activity. Such variants include allelic and non-allelic variants.

(i) Substitutions

An example of a variant within the scope of the present invention is a Protein C or Activated Protein C which has one or more amino acids substituted for the natural residues.

The skilled person is aware that various amino acids have similar properties. One or more such amino acids of a polypeptide can often be substituted by one or more other such amino acids without eliminating a desired activity of that polypeptide.

For example, the amino acids glycine, alanine, valine, leucine and isoleucine can often be substituted for one another (amino acids having aliphatic side chains). Of these

possible substitutions it is preferred that glycine and alanine are used to substitute for one another (since they have relatively short side chains) and that valine, leucine and isoleucine are used to substitute for one another (since they have larger aliphatic side chains which are hydrophobic).

Other amino acids that can often be substituted for one another include:
phenylalanine, tyrosine and tryptophan (amino acids having aromatic side chains);
lysine, arginine and histidine (amino acids having basic side chains);
aspartate and glutamate (amino acids having acidic side chains);
asparagine and glutamine (amino acids having amide side chains);
and cysteine and methionine (amino acids having sulphur containing side chains).

Substitutions of this nature are often referred to as "conservative" or "semi-conservative" amino acid substitutions.

(ii) Deletions

Amino acid deletions can be advantageous since the overall length and the molecular weight of a polypeptide can be reduced whilst still retaining activity. This can enable the amount of polypeptide required for a particular purpose to be reduced. For example, dosage levels can be reduced.

(iii) Insertions

Amino acid insertions relative to the natural Protein C or Activated Protein C sequence can also be made. This may be done to alter the properties of the polypeptide (e.g. to assist in identification, purification or expression, as explained above in relation to fusion proteins).

Polypeptides incorporating above amino acid changes (whether substitutions, deletions or insertions) can be provided using any suitable techniques. For example, a nucleic acid sequence incorporating a desired sequence change can be provided by site directed mutagenesis. This can then be used to allow the expression of a polypeptide having a corresponding change in its amino acid sequence.

One way of determining amino acid sequence identity is to align a given amino acid sequence with the amino acid sequence of natural Protein C or Activated Protein C in a manner which achieves the maximum number of matches of amino acids over the full length of the natural amino acid sequence (or a single chain of it). The percentage sequence identity will then be (**m**/**t**) x 100 , where **m** is the number of matches between the two aligned sequences over the length of the amino acid sequence in the natural sequence and **t** is the total number of amino acids present in the natural amino acid sequence.

Another way of determining sequence identity allows for the introduction of gaps when matching two amino acid sequences (0,1,2,3 or even more gaps may be allowed for in each sequence). This can be done, for example, by using the "Gap" program, which is available from Genetics Computer Group as part of "The Wisconsin Package". This program is based upon an algorithm provided by Smith and Waterman (*Advances in Applied Mathematics*, 482-489 (1981)).

Whatever the precise form of the molecule used in the invention, it will have significant Protein C or Activated Protein C activity, for example as determined by the experimental protocol in Example 4.

Most preferably, the natural form of Protein C or Activated Protein C is used. However, any variants or derivatives having the activity of Protein C may also be used.

If variants and derivatives are used, whatever amino acid changes may be made, preferred proteins of the present invention have at least 50% sequence identity with the natural amino acid sequence of Protein C; more preferably the degree of sequence identity is at least 75%. Sequence identities of at least 90% or of at least 95% are most preferred. Where high degrees of sequence identity are present there may be relatively few differences in amino acid sequence. Thus for example there may be fewer than 20, fewer than 10, or even fewer than 5 differences.

A medical device of the present invention includes any device used in medicine but is especially a device which comes into direct contact with blood. The contact with blood may be within a patient or extracorporeal, temporary or permanent. Accordingly, examples of such devices include, but are not limited to: vascular prostheses including stents, catheters, dialysis membranes, artificial hearts or components thereof, artificial heart valves and surgical suture material. The invention finds particular use in stents used in vascular angioplasty.

Such devices may be used in humans, or may be used in general veterinary industry, in particular domestic pets such as dogs and cats and farm animals such as horses, pigs, cattle, sheep, *etc.*

Stents which have been treated with Protein C according to the current invention gradually elute Protein C that retains potent anticoagulant activity. Such stents will obviate stent thrombosis following a variety of clinical thrombogenic situations, eg elective percutaneous coronary artery angioplasty. Local delivery of an inherently safe anticoagulant molecule in this way provides a safer alternative to currently used anticoagulant therapies. A drug-eluting stent provides the further advantage of not

causing additional vascular injury or myocardial ischaemia. It may also allow longer local drug retention (*Linkoff et al, 1994*)

To at least part of the surface of the device, Protein C or Activated Protein C is attached. Preferably, the area covered is all or substantially all of the area that comes into contact with blood. However, in some instances it may be desirable, for reasons of economy or otherwise, that only part of the area of a device is covered by the protein, the result of which is a device which nevertheless functions adequately for the purposes described above. Such a device is also within the scope of the present invention.

According to the present invention, Protein C (in various forms described above) is directly or indirectly attached to the surface of a device.

The protein can be attached onto the surface of the device directly, or indirectly through an intermediate layer. In the latter case, the intermediate layer can be of any suitable material known to the person skilled in the art, depending on the construction of the device. For example, polymeric coating, eg. using a nitrocellulose polymer, may be suitable for stents.

The attachment of the protein to the surface, or an intermediate layer, of a device can be by any method. The form of attachment is not critical as long as the biological function of the protein is not unduly affected. Accordingly, it includes physical attachment such as adsorption or absorption, as well as chemical bonding, ionic interaction, Van der Waals forces and other conventional means of attaching biological materials to objects.

In one embodiment, the protein is directly adsorbed onto an intermediate polymeric coating on the surface of a device.

The scope of the present invention also extends to devices, the surface to which one or more other molecules, which do not have the activity of Protein C but which may, for example, aid the activity of Protein C, are directly or indirectly attached. Thus, cofactors such as Protein S can also be attached to the surface of the device, along with proteins having the activity of Protein C. Other possible molecules are angiopeptin and forskolin. Devices with more than one type of Protein C molecule attached are also included in the scope of the present invention.

The present invention also provides a method of reducing the tendency of a medical device to cause blood coagulation when it is in direct contact with blood, the method comprising directly or indirectly attaching a protein having the activity of Protein C onto at least part of the surface of the device.

As described above, the method of attaching the protein may be physical or chemical. Preferably the attachment is by adsorption of the protein onto the surface of a device. However, the attachment may also be by chemical bonding.

The preferences described above for a device according to the present invention apply *mutatis mutandis* to the method.

The invention will now be illustrated using the following examples, which demonstrate the anti-thrombotic potential of polymer coated stents adsorbed with rabbit Activated Protein C. These examples are intended only to be illustrative should not be construed as in any way limiting. The examples refer to the accompanying drawings, in which:

Figure 1 is a chart showing the rate of adsorption of Activated Protein C onto pieces of stent-wire;

Figure 2 shows the elution of APC from APC-adsorbed wires (the quantity of APC remaining on stent-wires when placed in a perfusing circuit is shown);

Figure 3 shows the effect of APC-eluting wires on the deposition of fibrinogen/fibrin in a recalcified plasma system, wherein the black columns represent results obtained after 2 hours and the grey columns represent results obtained after 4 hours;

Figure 4 shows ^{125}I-Fibrinogen/fibrin deposition on stent-wires;

Figure 5 shows the effect of APC in an activated partial thromboplastin time assay;

Figure 6 shows the effect of APC-eluting wires on an activated partial thromboplastin time assay, wherein the diamonds represent group 1 results, the squares represent group 2 results and the triangles represent the averaged values;

Figure 7 shows activated partial thromboplastin time assay tested on ambient buffer containing stent-wires;

Figure 8 shows the flow variation recorded by probes set distal to stented segments, wherein probe A is APC-loaded stent; probe B is control stent; probe C is albumin-loaded stent; and *a* shows biphasic flow characteristic seen before stent depolyment; *b-c* show flow after 1 hour; and *d* shows flow in albumin loaded stent;

Figure 9 shows the percentage of flow remaining 2 hours after stenting; and

Figure 10 shows platelet deposition *in vivo* for various stent types.

General Methods

Cellulose polymer-coated stainless steel stent wires and stents (20 mm long x 3 mm expanded diameter) were provided by Cook Inc. Radioactive iodine (^{125}I) and indium (^{111}In) was purchased from Amersham Life Science (Bucks, UK). Citrated rabbit plasma for in vitro work was obtained from Harlan-Sera-Lab Ltd. (Crawley Down, UK). All other products were purchased from Sigma Chemicals Co (St Louis, MO)

unless otherwise stated. APC and rabbit fibrinogen (fraction 1) were labeled with ^{125}I by the Iodo-gen method. Autologous rabbit blood was drawn on the same day of operation and platelets ^{111}Indium-labelled as previously described by Aggarwal RK et al [Aggarwal RK, Ireland DC, Azrin MA, Ezekowitz MD, de Bono D, Gershlick AH. Antithrombotic potential of polymer-coated stents eluting platelet glycoprotein IIb/IIIa receptor antibody. *Circulation*. 1996;94:3311-3317]. All radioactivity was measured using the automated Cobra-II auto-gamma (Packard, CT). Animal procedures were conducted in accordance with the Animals [Scientific Procedures] Act 1986 under a licence from the Home Office, London.

Preparation of rabbit activated protein C

Protein C was isolated from citrated rabbit plasma using DEAE anion exchange chromatography. Bound proteins were eluted with calcium chloride. Following this, immunoaffinity chromatography was performed using the protein C-specific monoclonal antibody, HC2 from Sigma Chemicals Co (St Louis, MO). Elution of bound protein C was effected using alkaline pH.

Rabbit protein C was activated using activator protease purified from the venom of the Southern Copperhead snake *(Agkistrodon contortrix contortrix)* (ACC-C), which was a gift from Dr. Walter Kisiel (University of New Mexico, Alberquerque). ACC-C was purified away from protein C after activation using anion exchange chromatography on a Resource Q column (Amersham Pharmacia Biotech, Bucks, UK).

Using the protein C activation peptide-specific monoclonal antibody, HC2, and ACC-C-specific antibodies (a gift from Dr. Walter Kisiel, University of New

Mexico, Alberquerque), the rabbit protein C preparation was shown by Western blotting to be at least 95% activated and free of ACC-C.

Statistical analysis

Results are shown as either mean ± SD or as proportions. Differences between groups are analysed by one-way ANOVA. Where values could not be assumed as normal distribution, Kruskal-Wallis test was used. Where there was a significant overall difference between groups, multiple comparisons were made by Mann-Whitney U test. Differences in platelet deposition were analysed by one-sample t test. Significance was defined as $P<0.05$.

Example 1

Polymer-coated stent wire segments (10-mm lengths) were immersed in a 0.542 mg/mL solution of APC containing an iodinated-APC spike (specific activity 4.7 cpm/ng (or 4.46μCi/μg may be used) in 20 mM TBS/1%BSA buffer (pH 7.2 (or pH 7.4 may be used) at 37°C. APC solutions were contained in 1.5 mL polypropylene (Eppendorf) tubes. Total and uniform immersion of wire segments was ensured by placing the tubes on a Magic Roundabout Wheel. One, 2.5, 5, 10, 20, 30, 45, 60, 120 and 180 minutes after immersion, wires were removed from each solution rinsed 10 times in 5 mL PBS and gamma counted for 15 seconds (15-75 keV window for ^{125}Iodine; Cobra II auto-gamma counting system, Packard Instruments, Meridien, CT) to determine APC binding onto each wire. Six wires were assessed for each time point.

Upon immersion in APC solution [0.542 mg/mL] at 37°C, APC adsorption onto polymer-coated stent wires was shown to increase uniformly over time. Optimal and

stable adsorption appeared to be achieved after 60 minutes with 65.7 ± 9.6 ng of APC binding to 10-mm wire. Further immersion shown no further binding. (Figure 1)

Example 2

Stent wire segments were immersed in 0.542 mg/mL solutions of APC in buffer at 37^{0}C for 60 minutes as described above. Baseline protein binding to wires was determined by counting the radioactivity of each wire. The wires were then placed in individual housings in a closed-loop circuit continuously perfusing PBS/1%BSA at a rate of 10 mL/min (or a rate of 20ml/min may be used). Housings were made from glass chambers, diameter 2.0 mm, connected to a manifold device (modified from a multiple manifold dispenser; LabIndustries, CA) to ensure equal flow through across all channels. The PBS/1%BSA was pumped through the circuit with a peristaltic pump (Watson-Marlow 302S, Falmouth, UK). Sterile silicone tubing (3-mm bore, Fisons, Loughborough, UK) was used to carry the perfusate to the chambers housing the stent wires. At each time point of 10, 20, 40 minutes, 1, 2, 4, 18, 24 and 48 hours, all wires were removed from the circuit and individually gamma counted to quantify the amount of protein remaining bound to each. After counting, wires were returned to the circuit until the next time point. Six wires were used in the perfusing circuit.

In a perfusion circuit, APC was shown to elute off the loaded stent in a biexponential manner. 16.96+-3.43ng of APC remained on the stent wire after 24 hours. (Figure 2)

Example 3

(a) To study the effect of APC-eluting wires, a fibrinogen/fibrin deposition system was set up using recalcified plasma spiked with an iodinated label (^{125}I-fibrinogen; specific activity ≅ 2.32 μCi/μg). Wires optimally adsorbed with APC were prepared as above and placed in the recalcified plasma system. Wires were removed at 2 and 4h and counted for fibrinogen/fibrin deposition. Wires adsorbed with inhibited-APC (iAPC) and non-active protein (Alb) were used as controls. APC active-site was inhibited in this experiment using Phe-Pro-Arg Chloromethyl Ketone (PPACK) [54.2μg APC blocked with 3.5nmol PPACK]. Fibrinogen/fibrin deposition was shown to be significantly reduced on APC-eluting wires compared to wires adsorbed with non-active protein and wires adsorbed with inhibited-APC. At 2h fibrinogen/fibrin deposition was 1:8.5 ($p<0.01$, ANOVA) and 1:1.7 ($p=0.718$) for APC:iAPC and APC:alb respectively. At 4h, it was 1:21.7 ($p<0.0001$) and 1:4.7 ($p<0.0001$). (Figure 3)

(b) Stent wire segments were immersed in 0.542 mg/mL solutions of APC in buffer at 37^{0}C for 60 minutes as described above. Following ten times of rinsing with PBS/1%BSA, wires were placed in 200 μL of recalcified rabbit plasma containing an iodinated-fibrinogen spike (specific activity ≈2.32 μCi/μg). After incubation at 37^{0}C for 4 hours, wires were removed and counted to determine ^{125}fibrinogen/fibrin deposition. Comparison was made to controls consisting of (a) plain polymer-coated wire segments, (b) stainless steel wire segments, (c) polymer-coated wire segments similarly treated with inactivated-APC, (d) polymer-coated wire segments similarly treated with 10%BSA. APC was inactivated incubating with 200 μmol PPACK dihydrochloride (D-Phe-Pro-Arg Chloromethylketone HCl, Calbiochem,

Nottingham, UK) at 37^{0}C for 60 min. Six wire segments (10-mm lengths) were used for each group.

Because APC ultimately leads to the reduction of fibrin generation via feedback inhibition on thrombin generation, we set out to assess the effect of the APC-eluting stent wire on fibrin deposition in vitro. Stent wires were incubated in a recalcified plasma system containing an iodinated-fibrinogen spike for 4 hours. Relative ^{125}fibrinogen/fibrin deposition ratios after 4 hours were 1:5.27 (ANOVA, $P<0.001$), 1:53.8 ($P<<0.001$), 1:4.7 ($P<0.001$) and 1:2.63 ($P<0.001$) for APC-eluting wire:plain base-polymer wire, APC:stainless steel wire, APC:wire preloaded with 10%BSA and APC:wire preloaded with inhibited-APC respectively. In this plasma system, there was significantly less fibrinogen/fibrin deposition on APC-eluting stent wires compared to the other wire types. Deposition was significantly decreased on APC-loaded stents compared to inhibited-APC loaded stent, albumin-loaded stent, plain base polymer-coated stent and plain stainless steel stent. (Figure 4)

Example 4

The activity of the rabbit APC was tested in an activated partial thromboplastin time (APTT) assay using a rabbit plasma system. 100 μL citrated rabbit plasma, 100 μL PTT Automat 5® (Diagnostica Stago, Asnieres, Fr) and 50 μL 20mM pH7.2 TBS/1%BSA buffer containing increasing concentrations of APC were mixed in a 1-mL aggregometer tube containing a metal stirrer and incubated at 37^{0}C for 3 min. Clotting was initiated with 100 μL 20 mM $CaCl_2$. (Figure 5)

Example 5

(a) Using the APTT assay in separate experiments, wires optimally adsorbed with APC as above were immersed in 80µl PBS pH 7.2 and aliquots of the buffer were removed at 0, 2, 5, 10, 15, 45, 60, 90 and 120 min and put through APTT assays. In the APTT assay, it was shown that APC eluting from optimally coated wires continued to remain biologically active and prolonged clotting times significantly. (Figure 6)

(b) Stent wire segments were immersed in 0.542 mg/mL solutions of APC in buffer at 37^{0}C for 60 minutes as described above. Wires were then placed separately into tubes containing 80 µL TBS/1%BSA buffer. At 0, 2, 5, 10, 15, 45, 60, 90 and 120 minutes, 50 µL buffer was removed and tested in the APTT assay described above. Plain polymer-coated wires were used as negative controls. Four wire segments were used for each time point.

The in vitro APTT assay was simple to perform and reproducible. It demonstrated the potency of the rabbit APC sample (r^2=0.952). By taking buffer that was mixed with APC-eluting stent wires and testing the buffer in the APTT assay, clotting times were shown to be prolonged in a time-related manner (r^2=0.832). We conclude that eluted APC remains functional and potent after release from the stent-wire. (Figure 7)

Example 6: Efficacy of the APC-eluting stents in vivo

The in vivo efficacy of APC-eluting stents was tested in a rabbit iliac artery model of stent thrombosis previously described in Aggarwal RK et al [Aggarwal RK, Ireland DC, Azrin MA, Ezekowitz MD, de Bono D, Gershlick AH. Antithrombotic

potential of polymer-coated stents eluting platelet glycoprotein IIb/IIIa receptor antibody. *Circulation*. 1996;**94**:3311-3317]. New Zealand White male rabbits, weight range between 3.5 to 4.5 kg, were used. Each rabbit received aspirin 6 $mg.kg^{-1}.d^{-1}$ and Ticlopidine 8 $mg.kg^{-1}.d^{-1}$ administered in drinking water for 5 days and 3 days respectively prior to operation. 17 mL of blood was collected from an ear artery 2 hours before operation into 3 mL of acid-citrate-dextrose for ^{111}Indium-labeling of platelets. All animals were anaesthetised with Hypnorm 0.3 mL/kg IM (fentanyl citrate, 0.315 mg/mL and fluanisone, 10 mg/mL) and inhaled halothane and oxygen. Animals were placed on a heating pad (38^0C) and had continuous intraoperative monitoring of rectal temperature, heart rate and respiratory rate.

APC-eluting stents were prepared by immersion of stents into either 1.24 mg/mL or 0.62 mg/mL of APC solutions (under the in vitro conditions described above). Plain polymer-coated stents and stents similarly loaded with 1.2mg/mL BSA were used for controls. The type of stent deployed was assigned with the operator blinded.

Operation procedure

One hour before induction of anaesthesia, each animal was given the autologous injection of ^{111}In-labeled platelets (3-mL suspension, 0.15 to 0.27 mCi) via an ear vein. At operation, bilateral femoral and common iliac arteries and the abdominal aorta were exposed. After allowing flow to stabilise for 10 min following dissection, baseline blood flow was recorded for 2 to 3 minutes in each vessel using two separate perivascular transit time flowprobes (T206 small animal blood flow meter with 2.5SB probes; Transonic Inc, Ithaca, NY). A superficial femoral artery arteriotomy was fashioned as distally as possible on each side. Through the arteriotomy, a 3-mm-diameter noncompliant coronary angioplasty balloon catheter

was first advanced to the common iliac artery under direct vision. The balloon was then inflated three times to 8 atm for 60 seconds with 60 seconds interval between each inflation to cause deep vessel wall damage. Next, the balloon-mounted stent (prepared as above) was deployed at 6 atm to the site of arterial injury via the arteriotomy. After removal of balloon, a ligation of the arteriotomy site was made to reduce blood flow through the stent. The same was done for the contralateral artery. Animals received either an APC-eluting stent or a stent adsorbed with BSA [1.2 mg/mL] in one artery and a plain polymer-coated stent in the contralateral artery. After stent implantation, flow was measured continuously for 2 hours with the perivascular flowprobes placed just distal to the stented segments.

More than half of the arteries (9/15) implanted with control plain polymer-coated stents completely occluded during the 2-hour period of monitoring (Table 1).

Table 1. Results showing the outcome of stented arteries in vivo.

	No of occluded arteries/total number studied
APC-loaded [1.24mg/mL]	0/8
APC-loaded [0.62mg/mL]	0/6
Albumin-loaded [1.2mg/mL]	2/4
Plain polymer-coated	9/15

The ones that occluded did so within 30 minutes following stent deployment. Those that did not occlude after 2 hours continued to sustain flow at extremely low flowrates (0.2-0.56 mL/min). Residual flow through these arteries was non-pulsatile

and non-biphasic (Figure 8b, c, d) There was a consistent and dramatic drop in flowrates within 30 minutes of deploying a control stent.

APC eluting stents (3 x 20mm, balloon-mounted) were prepared for the study by complete immersion of plain polymer-coated stents in APC solution [either 1.24 mg/mL or 0.62 mg/mL] at 37^{0}C pH 7.5 for 60 minutes. Each immersion was undertaken just prior to stent deployment.

None of the APC-eluting stents occluded throughout the 2-hour period of monitoring (0/8 for 1.24 mg/mL and 0/6 for 0.62 mg/mL). Significantly a pulsatile and bi-phasic flow characteristic was maintained over the 2 hour monitoring period in these stents (Figure 8b, c). Moreover, 2 animals were monitored for a further one-hour and in these animals the arteries remained unoccluded up to the end of the 3-hour period.

There was no statistically significant difference in flow immediately after distal ligation across arteries of control stents, APC stents and albumin-loaded stents (3.51 ± 1.9 mL/min; K-W, P=0.37). Percentage of remaining flowrate 2 hours after stent deployment however was statistically significantly higher in APC stents as compared to control stents (M-W, P<0.001) and higher in APC stents as compared to albumin-loaded stents (P<0.001). There was no statistically significant difference between the outcomes of APC stents loaded at the 2 different concentrations of 1.24 mg/mL and 0.62 mg/mL (P=0.08). The average percentage of flow remaining in APC-eluting stents was 45.81 ± 8.8% for 1.24 mg/mL and 44.86 ± 13.9% for 0.62 mg/mL. The average percentage of flowrate remaining in albumin-loaded stents was 3.10 ± 3.7%; and that for plain polymer coated stents was 2.53 ± 3.4% (Figure 9).

Albumin-loaded stents were prepared by immersion of polymer-coated stents in BSA [1.2 mg/mL] 37^{0}C for 60 minutes. Flowrate changes in arteries deployed with albumin loaded stents were similar to that in plain polymer coated stents. Flowrates were dramatically reduced within 30 minutes of stent deployment and remained non-pulsatile and non-biphasic in the arteries that did not occlude (Figure 8d).

Example 7: Platelet deposition in stented arteries

At the end of the above experiment, animals were killed with an overdose of pentobarbitone (140 mg/kg). Arteries were then flushed with PBS and removed. Both stented vessels were counted in the automated gamma counter and relative platelet deposition determined by the ratio of radioactivity of one stented artery to the other. Each stented artery was weighed and the counts normalised according to the weight of the segment.

Platelet deposition was determined by counting ^{111}In-labeled platelet accumulation on the stented artery. For each animal relative platelet deposition was determined by the ratio of radioactivity on the study stented vessel compared to control stented vessel. The results reflected closely thrombosis that was observed in the arteries. APC-eluting stents had significantly reduced platelet deposition as compared to both albumin-loaded stents and plain polymer coated stents. Relative platelet deposition was 0.45:1 ($P<0.005$), 0.64:1 ($P=0.03$) and 1.15:1 ($P=0.12$) for APC stents [1.24 mg/mL], APC stents [0.62 mg/mL] and albumin-loaded stents respectively in comparison to plain polymer-coated stents (Figure 10). Platelet deposition on albumin-loaded stents and control stents were not significantly different. APC stents loaded at the lower concentration [0.62 mg/mL] demonstrated a wider variation in the degree of platelet deposition.

Example 8: Rabbit bleeding time and clotting assays

We evaluated the haemostatic parameters during the in vivo experiments by using two different methods. Uniform incisions 10 mm long and 1 mm deep were made on the ventral surface of the rabbit's ear avoiding the superficial veins. The same operator performed each incision on all the animals tested. Blood was blotted with filter paper (Whatman No.4) every 30 seconds avoiding the incision. Bleeding time was recorded as the interval between time of incision and time when blood did not stain the paper.

3-mL venous blood was removed at 30-minute interval during the 2-hour monitoring period, collected into 0.3-mL acid-citrate, centrifuged immediately and tested in the APTT assay.

None of the rabbits in the study had any increase in bleeding time when tested by the superficial ear incision method described above. APTT assays done on blood collected when animals were undergoing operations did not show any increase in clotting time (72.8 ± 5.5 s).

Example 9: Systemic Distribution of APC

In an animal where only one iliac artery was stented, the stent was pre-immersed expanded in an APC solution [1.2 mg/mL] containing an iodinated-APC spike. The animal was killed 2 hours after stenting and tissue/organs including the heart, aorta, liver, spleen, left lung, left kidney, urine, blood, stented artery and contralateral unstented artery were removed and gamma-counting to determine the systemic distribution of APC eluting off the stent. Each specimen was weighed and counts were normalised for weights.

In the animal receiving a stent loaded with APC containing a radioactive tracer, organs removed after 2 hours of monitoring, including the heart, aorta, liver, spleen, left lung, left kidney, urine, blood and the contralateral unstented artery, showed only background radioactivity as compared to the high radioactivity found on the stented artery.

REFERENCES

Arnljots et al, [1994] *Thrombosis and Haemostasis* **72**, pp415-420.

Bamford et al, [1983] *Eur Polym J*, **19**, pp1027-1035.

Breckwoldt et al, [1991] *J. Invest Surg*, **4** , pp269-278.

Colombo et al, [1995] *Circulation*, **91**, pp1676-1688.

Comp PC and Esmon CT, [1981] "Generation of Fibrinolytic activity by infusion of Activated Protein C into dogs." *J. Clin. Invest.*, **68** , pp1221-1228.

Comp PC and Esmon CT, [1979] *Blood*, **54** , pp1272-1281.

Cox et al, [1992] *Coronary Artery Disease*, **3**, pp237-248.

Dahlback and Stenflo, [1980] *Europ J Biochem*, **107** , pp331-335.

De Scheerder et al, [1993] *Circulation*, **88** (Suppl 1) , pp1-645.

Dichck et al, [1989] *Circulation*, **80** , pp1347-1353.

Dussaillant et al, [1995] *J Am Coll Cardiol*, **26**, pp720-724.

Esmon CT et al, [1976] *J. Biol Chem*, **251** , pp3052-3056.

Fischman et al, [1994] *New Engl J Med*, **331**, pp496-501.

Forrester et al, [1991] *J Am Coll Cardiol*, **17**, pp758-769.

Gibbons et al, [1994] *N Engl J Med*, **33**, pp1431-1438.

Grinnell et al, [1994] *Glycobiol*, **4** , pp221-225.

Grode et al, [1969] *Trans Am Soc Art Intern Organs*, **15** , pp1-5.

Grüber et al, [1989] *Blood*, **73**, pp639-642.

Grüber et al, [1990] *Circulation*, **82**, pp578-585.

Grüber et al, [1991] *Circulation*, **84**, pp2454-2462.

Grüber et al, [1993] *Lancet*, **342**, pp1275-1276.

Itakwa et al, [1977] *Science*, **198**, pp105-63.

Jozefowicz and Jozefowicz, [1986] Polymers in Medicine, Ed Chicilini et al, New York, Plenum Press.

Kisiel et al, [1977] *Biochem*, **16**, pp5824-5831.

Kisiel, W, [1979] *J. Clin Invest*, **64**, pp761-769.

Lambert et al, [1994] *Circulation*, **90**, pp1003-1011.

Libby et al, [1997] *Prog in Cardiovasc Dis*, **40**, pp97-106.

Linkoff et al, [1994] *Circulation*, **90**, pp207-208.

Mintz et al, [1996] *Circulation*, **94**, pp35-43.

Okajima et al, [1990] *Thrombosis and Haemostatis*, **63**, pp48-53.

Owen and Esmon, [1981] *J. Biol Chem*, **256**, pp5532-5535.

Sakamoto et al, [1994] *Circulation*, **90**, pp427-432.

Seegers et al, [1976] *J. Biol Chem*, **251**, pp353-355.

Serruys et al, [1994] *N Engl J Med*, **331**, pp489-495.

Snow et al, [1991] *Circulation*, **84**, pp293-299.

Stenflo, J., [1976] *Thrombosis Res.*, **8**, pp543-552.

Tanazawa et al, [1973] *Trans Am Soc Art Intern Organs*, **19** , pp188-194.

Van Beusekom et al, [1993] *Circulation*, **88**(Suppl 1), pp1-645.

Van der Giessen et al, [1992] *J Interven Cardiol*, **5**, pp175-185.

Walker et al, [1979] *Biochimica et Biophysica Acta*, **571** , pp333-342.

Williams et al, [1997] *Heart*, **77** (Suppl 1) , pp151.

CLAIMS

1. A medical device to at least part of the surface of which a protein having the activity of Protein C or Activated Protein C is directly or indirectly attached.

2. A device as claimed in claim 1 which is a vascular prosthesis, such as a stent, catheter, dialysis membrane, an artificial heart or a component thereof, an artificial heart valve or surgical suture material.

3. A device as claimed in claim 1 or 2 wherein the protein is attached onto the surface of the device indirectly, through an intermediate layer.

4. A device as claimed in claim 3 wherein the intermediate layer is a layer of polymeric coating.

5. A device as claimed in any of the above claims wherein the protein is attached by adsorption.

6. A device as claimed in any of the above claims wherein one or more other molecules are directly or indirectly attached.

7. A method of reducing the tendency of a medical device to cause blood coagulation when it is in direct contact with blood, the method comprising directly or indirectly attaching a protein having the activity of Protein C onto at least part of the surface of the device.

8. The use of a protein having the activity of Protein C or Activated Protein C in the preparation of a medical device which, when in contact with blood, discourages blood coagulation.

9. The use of a protein having the activity of Protein C or Activated Protein C in the preparation of a medical device, wherein the device is as defined in any one of claims 1 to 6.

10. A method of reducing the risk of blood coagulation in a system in which a medical device is, in use, in direct contact with blood, wherein a protein having the activity of Protein C is directly or indirectly attached onto at least part of the surface of the device, the method comprising allowing the device to come into contact with blood.

11. A method as claimed in claim 10, wherein the system is a cardiovascular system of a mammal.

12. A method as claimed in claim 10 or 11, wherein the mammal is a human.

13. A method as claimed in claim 10 or 11, wherein the mammal is a farm animal.

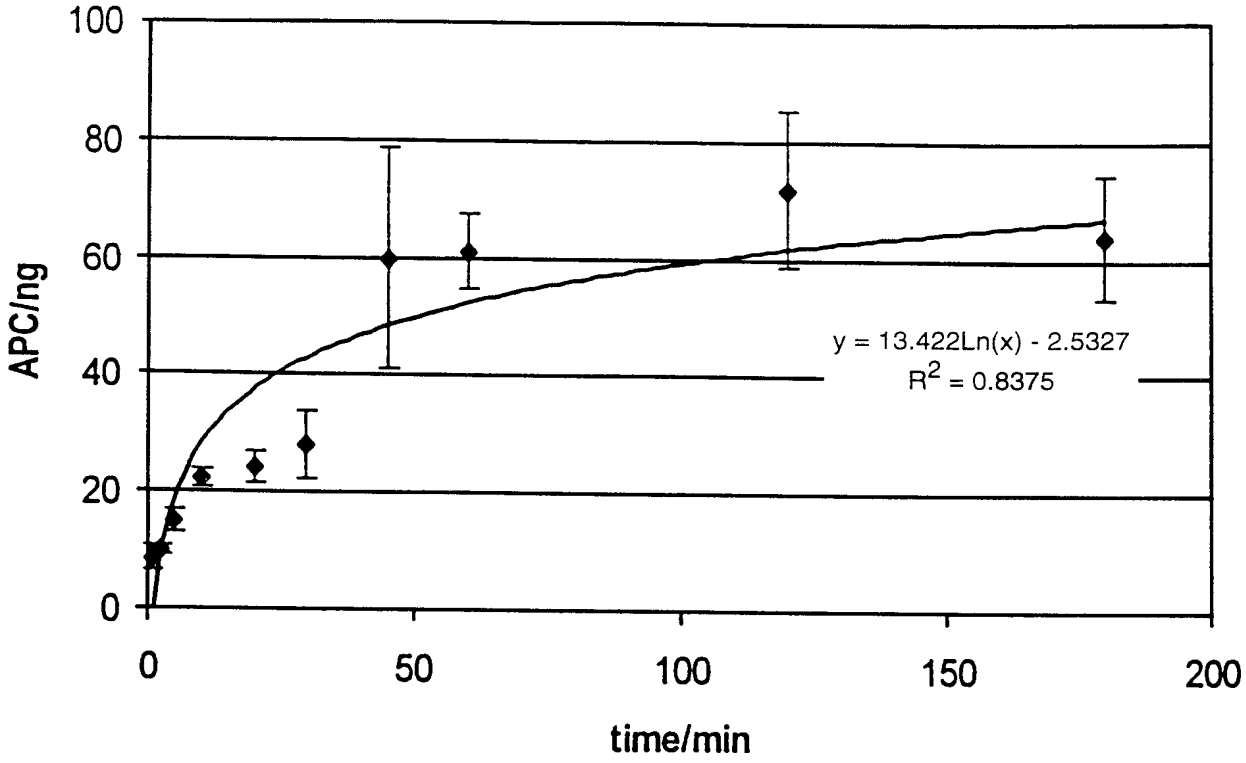
100
80
60
40
20
0
APC/ng
0
50
100
150
200
time/min
y = 13.422Ln(x) - 2.5327
R2 = 0.8375

FIG.1

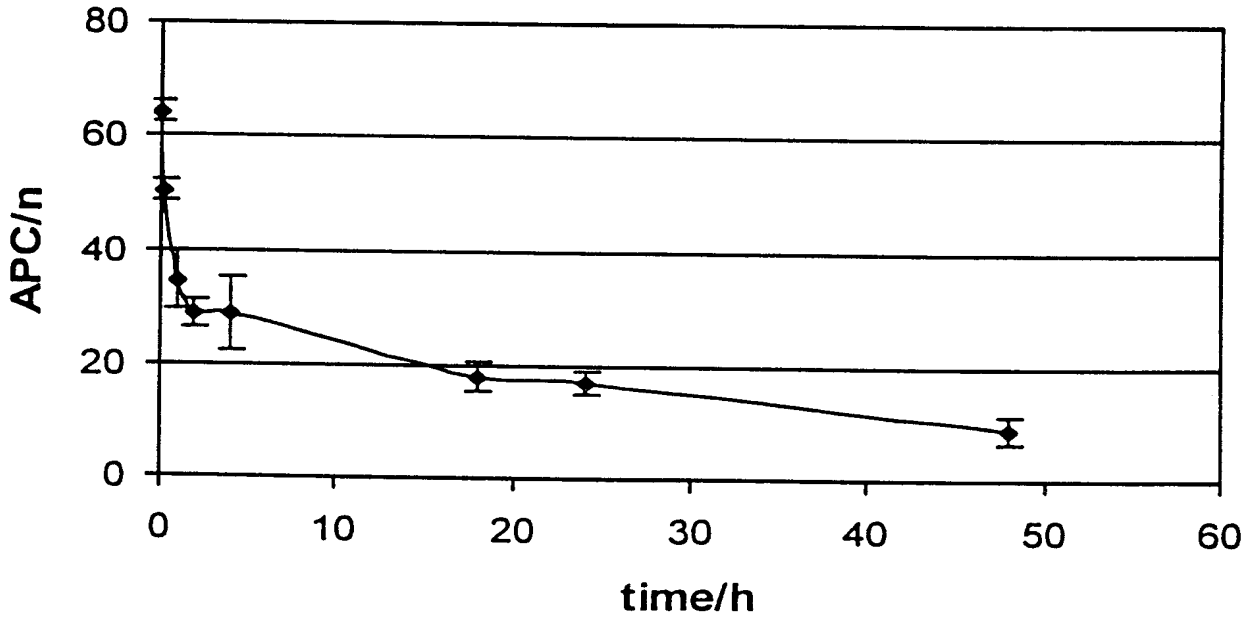
80
60
40
20
0
APC/n
0
10
20
30
40
50
60
time/h

FIG.2

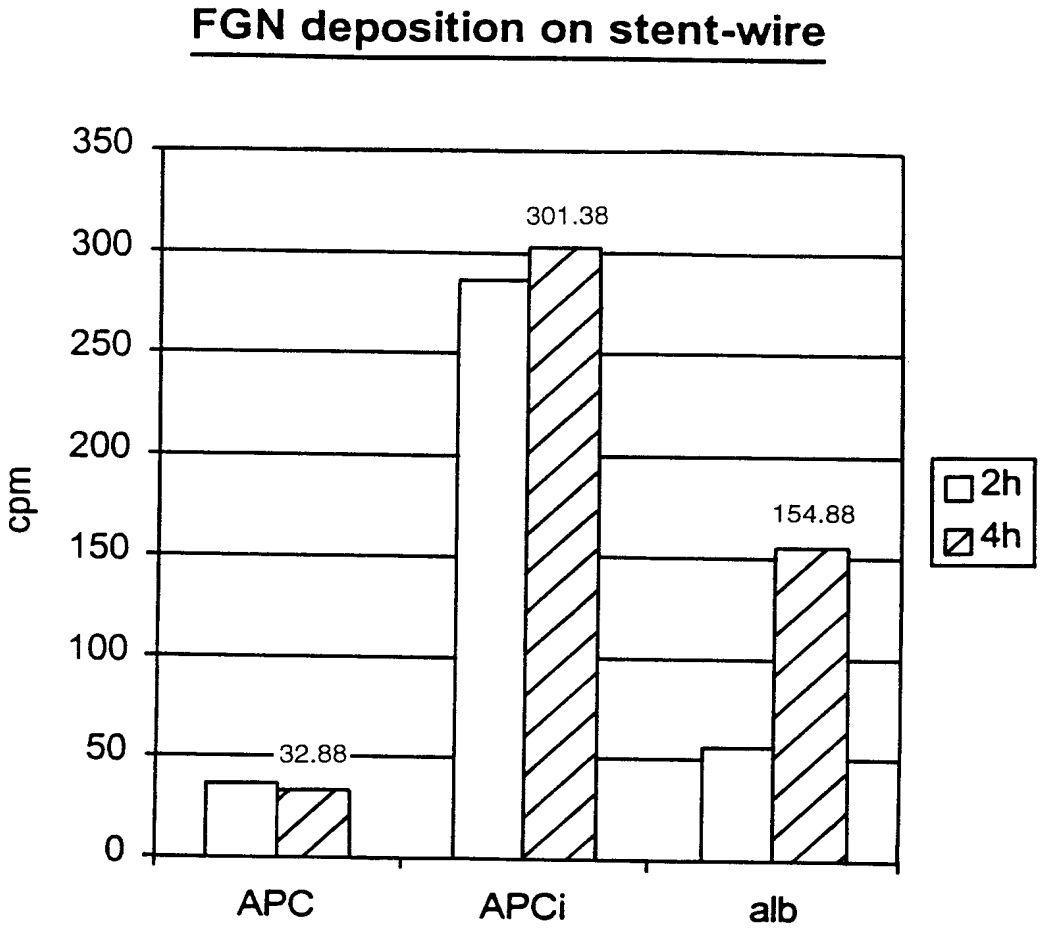
FGN deposition on stent-wire
350
300
250
200
150
100
50
0
cpm
301.38
154.88
32.88
2h
4h
APC
APCi
alb

FIG.3

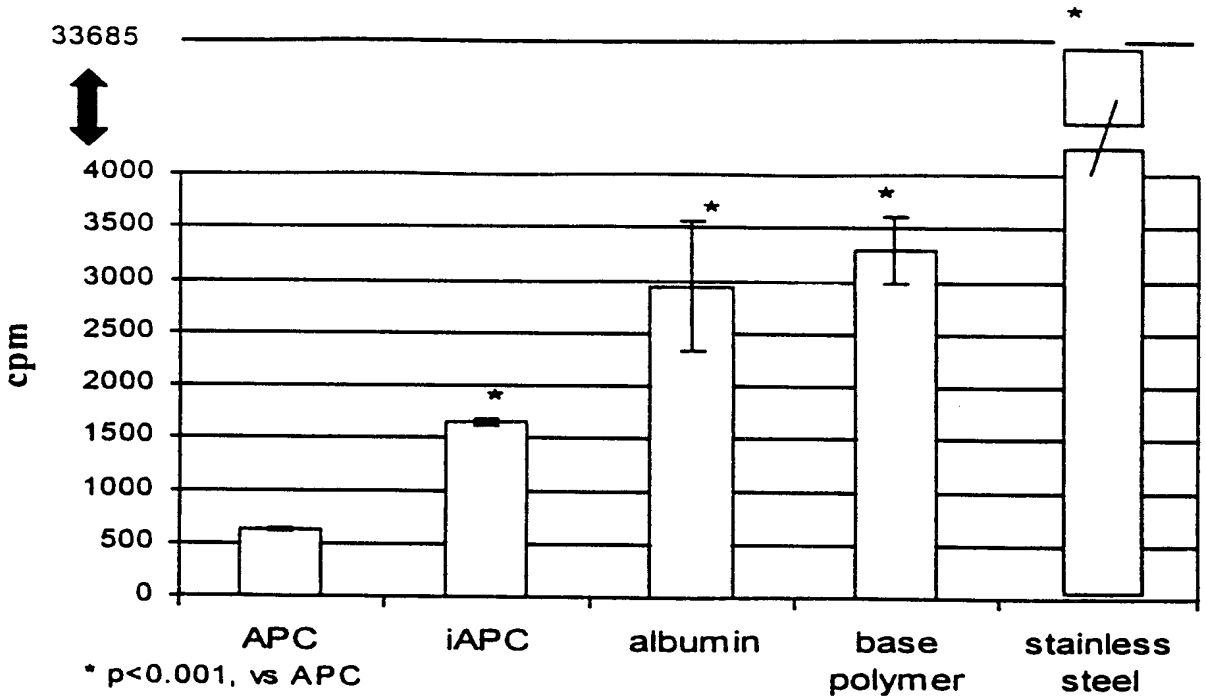
33685
4000
3500
3000
2500
2000
1500
1000
500
0
cpm
APC
iAPC
albumin
base polymer
stainless steel
* p<0.001, vs APC

FIG.4

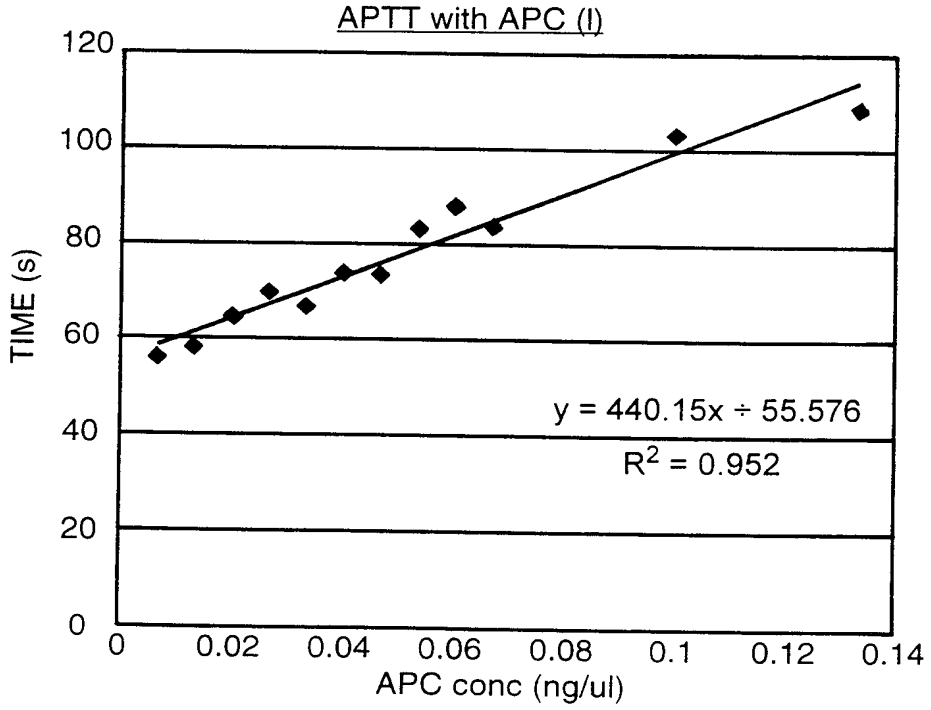
APTT with APC (I)
120
100
80
60
40
20
0
TIME (s)
y = 440.15x ÷ 55.576
R2 = 0.952
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
APC conc (ng/ul)

FIG.5

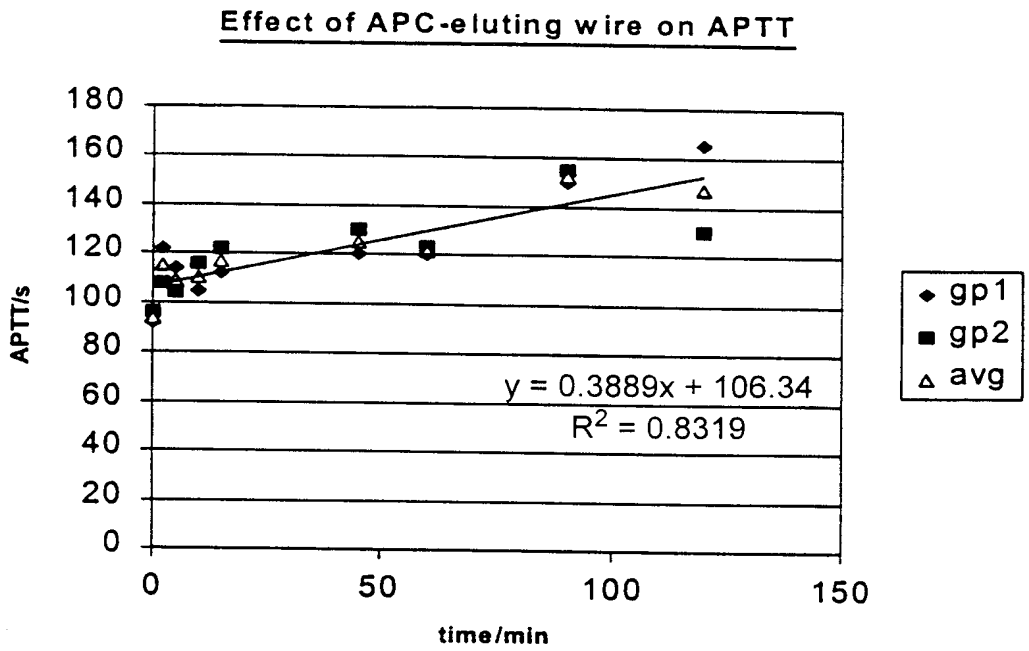
Effect of APC-eluting wire on APTT
APTT/s
180
160
140
120
100
80
60
40
20
0
0
50
100
150
time/min
y = 0.3889x + 106.34
R2 = 0.8319
gp1
gp2
avg

FIG.6

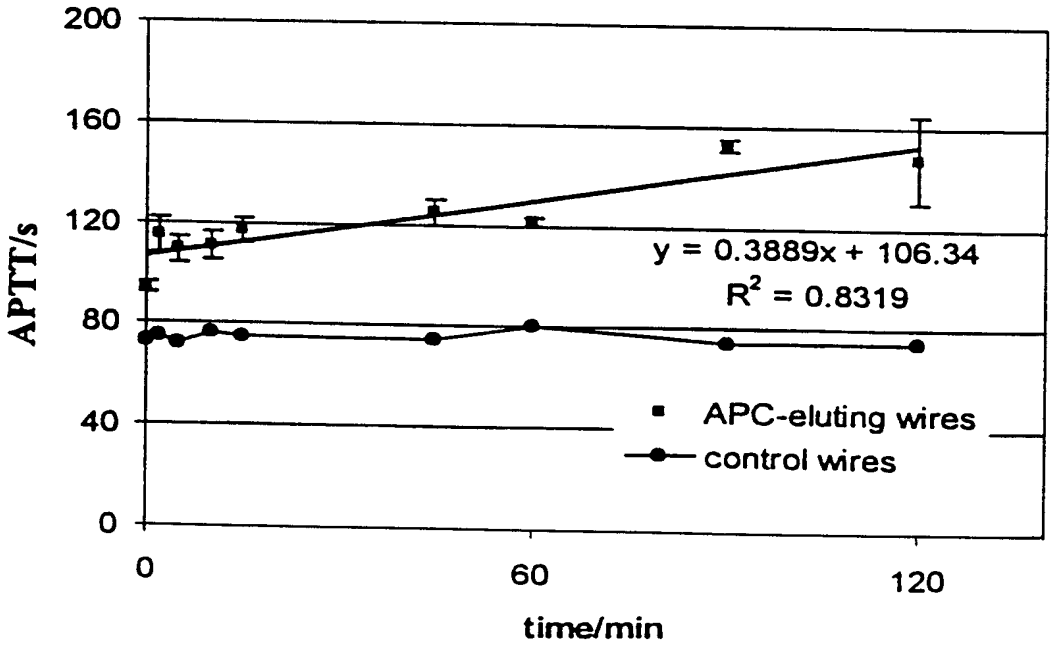
APTT/s
200
160
120
80
40
0
0
60
120
time/min
y = 0.3889x + 106.34
R2 = 0.8319
APC-eluting wires
control wires

FIG.7

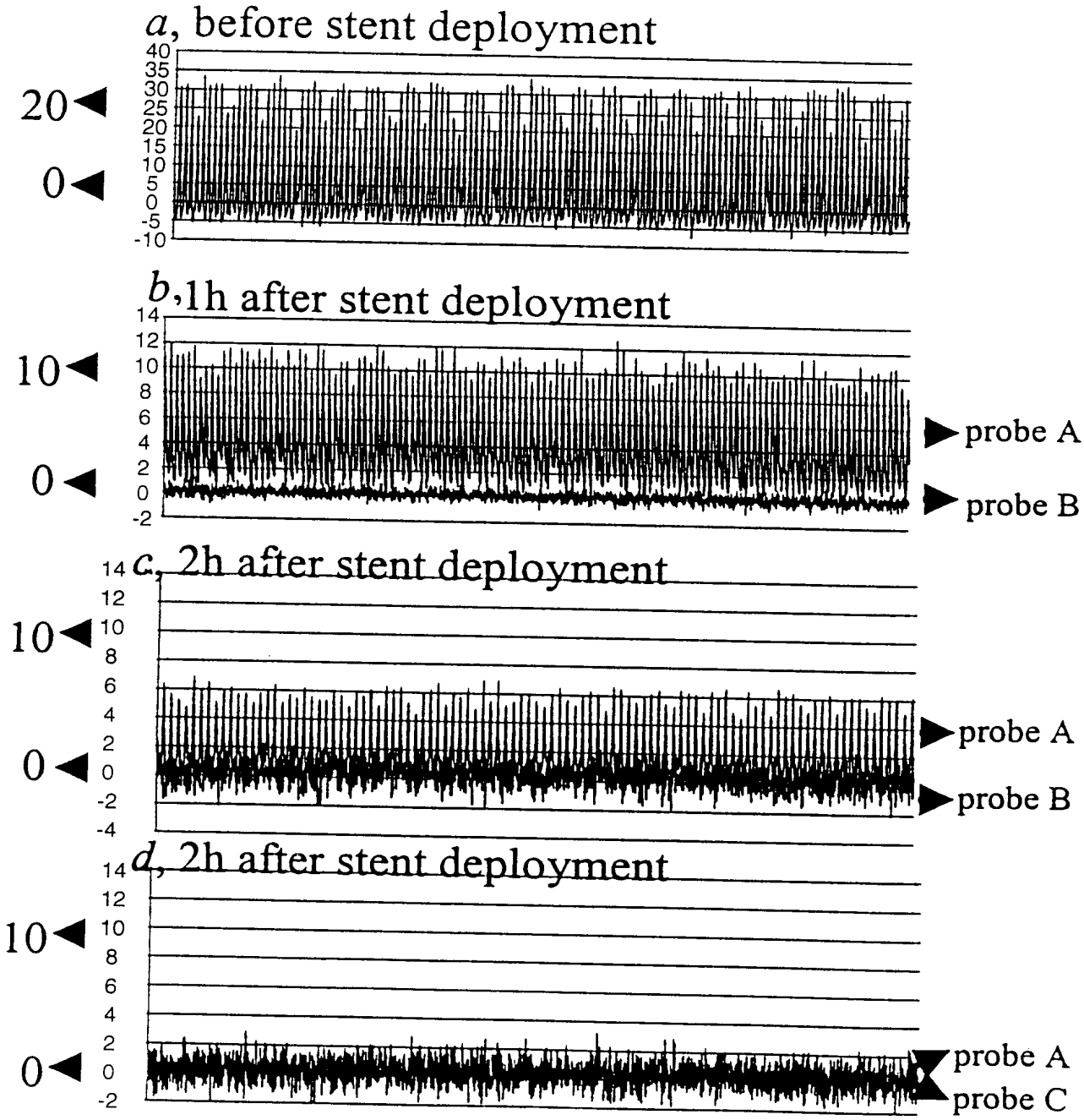
a, before stent deployment
20
0
40
35
30
25
20
15
10
5
0
-5
-10
b, 1h after stent deployment
10
0
14
12
10
8
6
4
2
0
-2
probe A
probe B
c, 2h after stent deployment
10
0
14
12
10
8
6
4
2
0
-2
-4
probe A
probe B
d, 2h after stent deployment
10
0
14
12
10
8
6
4
2
0
-2
probe A
probe C

FIG.8

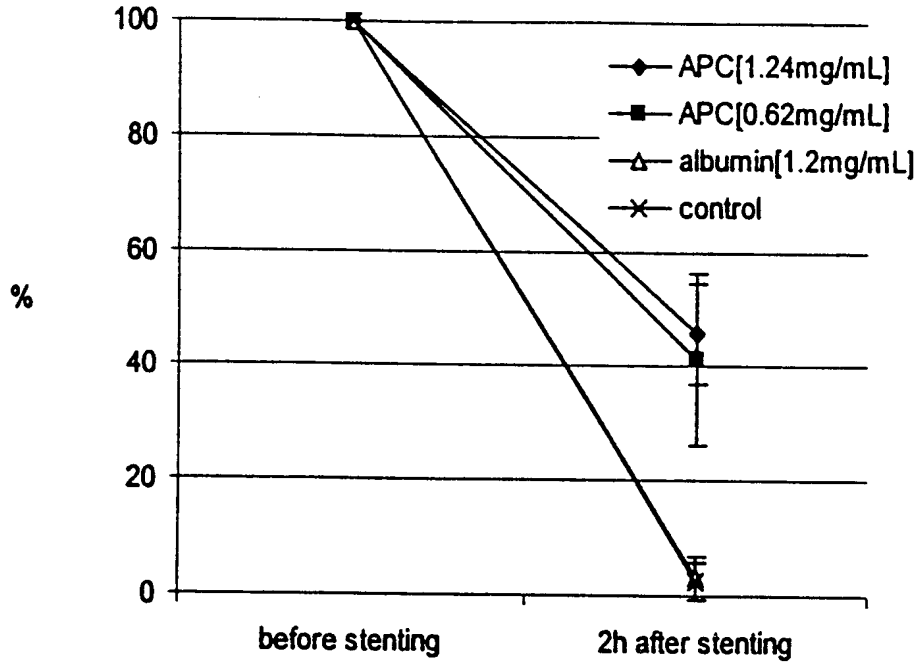
100
80
60
40
20
0
%
APC[1.24mg/mL]
APC[0.62mg/mL]
albumin[1.2mg/mL]
control
before stenting
2h after stenting

FIG.9

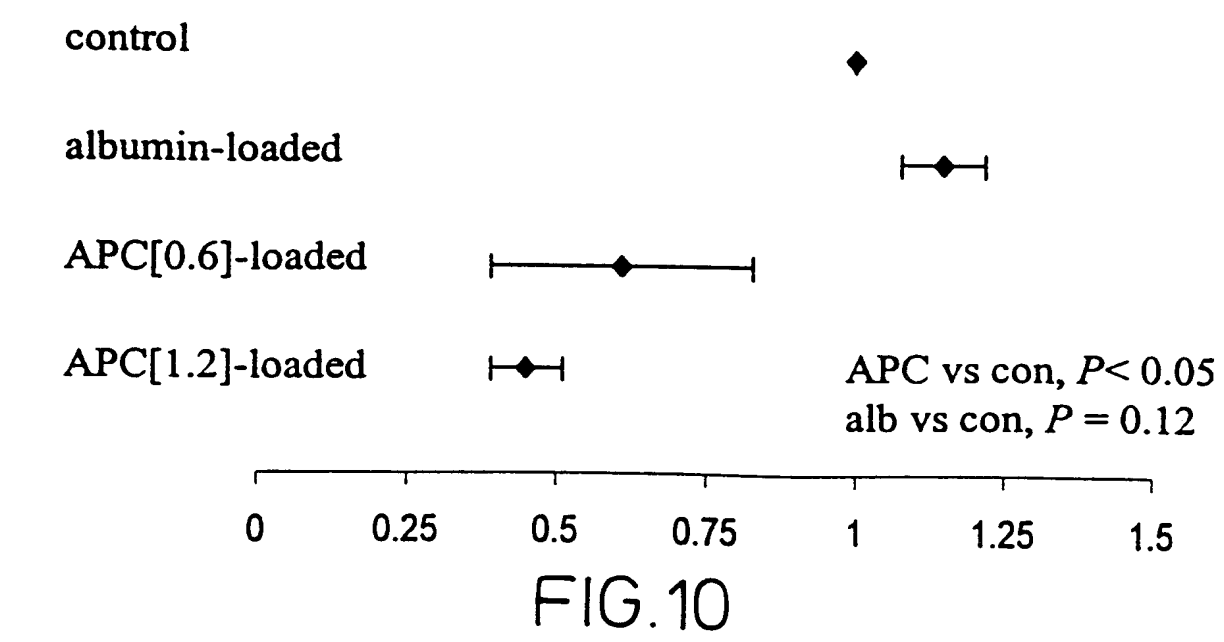
STENT TYPE
control
albumin-loaded
APC[0.6]-loaded
APC[1.2]-loaded
APC vs con, P< 0.05
alb vs con, P = 0.12
0
0.25
0.5
0.75
1
1.25
1.5

FIG.10

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/01008

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61L27/00 A61L29/00 A61L31/00 A61L33/00 A61L17/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 693 293 A (TERUMO CORP) 24 January 1996 (1996-01-24) page 8, line 39 - line 56 ---	1-13
X	WO 97 46590 A (GORE ENTERPRISE HOLDINGS INC) 11 December 1997 (1997-12-11) page 9, line 34 - page 10, line 2 ---	1-13
X	US 5 670 558 A (ISHII NAOKI ET AL) 23 September 1997 (1997-09-23) column 11, line 40 - line 55 ---	1-13
X	EP 0 512 122 A (TORAY INDUSTRIES) 11 November 1992 (1992-11-11) column 7, line 2 - line 12 ---	1-13

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 July 1999	30/07/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ESPINOSA, M

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/01008

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 46267 A (GORE ENTERPRISE HOLDINGS INC) 11 December 1997 (1997-12-11) page 8, line 28 - line 31	1-13
X	PATENT ABSTRACTS OF JAPAN vol. 014, no. 246 (C-0722), 25 May 1990 (1990-05-25) & JP 02 068039 A (KURARAY CO LTD), 7 March 1990 (1990-03-07) abstract	1
X	DATABASE WPI Section Ch, Week 8623 Derwent Publications Ltd., London, GB; Class A96, AN 86-147562 XP002109924 & JP 61 082760 A (MIURA Y), 26 April 1986 (1986-04-26) abstract	1
X	PATENT ABSTRACTS OF JAPAN vol. 016, no. 168 (C-0932), 22 April 1992 (1992-04-22) & JP 04 015063 A (MITSURU AKASHI;OTHERS: 02), 20 January 1992 (1992-01-20) abstract	1
A	EP 0 629 407 A (IMMUNO AG) 21 December 1994 (1994-12-21)	

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/01008

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0693293 A	24-01-1996	JP 8019598 A	23-01-1996
		JP 8019599 A	23-01-1996
		JP 8024327 A	30-01-1996
		JP 8024328 A	30-01-1996
		JP 8033704 A	06-02-1996
		CA 2153466 A	08-01-1996
		US 5670558 A	23-09-1997
WO 9746590 A	11-12-1997	US 5914182 A	22-06-1999
		US 5874165 A	23-02-1999
		AU 3229797 A	05-01-1998
		EP 0910584 A	28-04-1999
		US 5897955 A	27-04-1999
US 5670558 A	23-09-1997	JP 8019598 A	23-01-1996
		JP 8019599 A	23-01-1996
		JP 8024327 A	30-01-1996
		JP 8024328 A	30-01-1996
		JP 8033704 A	06-02-1996
		CA 2153466 A	08-01-1996
		EP 0693293 A	24-01-1996
EP 0512122 A	11-11-1992	CA 2074362 A	23-05-1992
		WO 9209312 A	11-06-1992
		US 5632776 A	27-05-1997
WO 9746267 A	11-12-1997	AU 3229897 A	05-01-1998
		EP 0918550 A	02-06-1999
JP 02068039 A	07-03-1990	JP 1794875 C	28-10-1993
		JP 4078295 B	10-12-1992
JP 61082760 A	26-04-1986	NONE	
JP 04015063 A	20-01-1992	NONE	
EP 0629407 A	21-12-1994	DE 4320294 A	22-12-1994
		AU 680894 B	14-08-1997
		AU 6471994 A	22-12-1994
		CA 2126135 A	19-12-1994
		CZ 9401482 A	18-01-1995
		FI 942941 A	19-12-1994
		HU 67074 A	30-01-1995
		JP 7048275 A	21-02-1995
		NO 942298 A	19-12-1994
		SK 74894 A	10-05-1995
		US 5614493 A	25-03-1997